NCI ETI Branch
Flow Cytometry Core Laboratory

Preparation of biotin- and fluorochrome-conjugated antibodies using amine-reactive N-hydroxysuccinimidyl esters

Although amine modification using succinimidyl esters (SE) has traditionally been used for biotinylation, many fluorochrome SEs are now available for protein conjugation. SE amine modification is more reproducible than isothiocyanate coupling (traditionally used to conjugate fluorescein and tetramethylrhodamine to proteins) and is now used more frequently for this purpose. This protocol can be used to attach fluorescein, tetramethylrhodamine, 7-amino-3-methylcoumarin acetic acid (AMCA) and biotin to most human and rodent antibodies. The following biotin- and fluorochrome-NHS can be used (but is not an exhaustive list):

- **5-carboxyfluorescein, succinimidyl ester** (Molecular Probes, C2210)
- **6-carboxyfluorescein, succinimidyl ester** (Molecular Probes, C-6164)
- **6-(fluorescein-5-carboxamido)hexanoic acid, succinimidyl ester** (Molecular Probes, F-2181)

  The third form of fluorescein SE in this list has a 7-atom spacer arm, allowing conjugations where steric hinderance is a problem.

- **Oregon Green 488 carboxylic acid, succinimidyl ester** (Molecular Probes, O-6146 and O-6148)

  A relatively new fluorochrome with excitation/emission characteristics similar to fluorescein. Less prone to photobleaching.

- **5-carboxytetramethylrhodamine, succinimidyl ester** (Molecular Probes, 6121)

  A bright fluorochrome similar to TRITC.

- **6-((7-amino-4-methylcoumarin-3-acetyl)amino)hexanoic acid, succinimidyl ester (AMCA-X)**(Molecular Probes, A-6118)
- **7-amino-3-((((succininimidyl)oxy)carbonyl)methyl)-4-methylcoumarin-6-sulfonic acid (AMCA-S, Alexa 350)** (Molecular Probes A-6120)

AMCA-X is the traditional SE for AMCA conjugations. We have had better luck with the AMCA-S sulfo SE form, which has higher water solubility.

- **6-((biotinoyl)amino)hexanoic acid, succinimidyl ester (biotin-X)** (Molecular Probes, B-1582 or Sigma B-2643)
- **6-((6-((biotinoyl)amino)hexanoyl)amino)hexanoic acid, succinimidyl ester (biotin-XX)** (Molecular Probes B-1606)

These forms of biotin SE also have spacer arms and are recommended for biotinylations with low steric hinderance. Sulfo SE forms of the above reagents (Molecular Probes B-6353 and B-6352 respectively) with higher water solubility are also available, as are chromogenic derivatives that allow easier chromatographic purification.

NHS forms of Texas Red, BODIPY, etc. can also be used. Many companies sell kits to couple fluorochromes to antibodies and other proteins by SE amine modification. SE forms of the Cy fluorochromes (such as Cy3 and Cy5) can be coupled to proteins using linker kits from Amersham and Bachem. Amine-reactive Cy5 can also be obtained from Fluka.

Procedure

- Antibodies to be conjugated should at a minimum be purified over a Protein A- or Protein G-Sepharose column prior to any conjugation reactions. Affinity-purified antibodies are ideal. Antibodies should be at an approximate protein concentration of at least 1 mg/ml total protein for conjugation. Total volume of antibodies should be between 0.5 and 2 mls. Larger volumes of antibodies often show reduced amine modification.

- Dialyze the antibody prep against 0.1 M sodium bicarbonate buffer pH 8.5 for at least 4 hours prior to conjugation.

- Dissolve the biotin- or flurochrome-NHS ester at 1 mg/ml in dry DMSO. All NHS-ester solutions should be made immediately prior to the conjugation process, as solutions of these compounds will gradually hydrolyze in solution. Use high-grade DMSO (water-free) - DMSO stored over molecular sieve to prevent water accumulation is recommended.

- Remove the antibody from dialysis and transfer to a 1.5 ml Eppendorf tube. With constant gentle vortexing, slowly add 125 microliters of the NHS ester solution to every 1 ml of antibody. This constitutes an 8:1 w/w ratio of antibody to biotin or fluorochrome. This ratio has been empirically determined for a variety of human and rodent antibodies and is a good starting point for determining the optimal protein:fluorochrome ratio. Ratios from 4:1 to 10:1 may need to be tested to obtain the optimal fluorochrome:proteins ratio.

- Incubate at room temperature for four hours with periodic mixing.

- Transfer the reaction mixture to dialysis tubing and dialyze against three 1000-fold volumes of PBS pH 7.4 at 4^{o}C to completely remove unreacted biotin or fluorochrome. Alternately, the antibody can be separated on a Bio-Rad BioGel column (if the fluorochrome is chromogenic). The fluorochrome-antibody conjugate will elute first.

- Determine the ratio of protein to fluorochrome spectrophotometrically. For **fluorescein conjugations**, this can be done using a spectrophotometer. Measure the absorbance of the conjugate at 280 nm (the protein concentration) and 495 (the fluorescein concentration).

  1 mg/ml IgG = absorbance of 1.4 at 280 nm

  1 mM fluorescein = 68 at 495 nm and 11.9 at 280 nm

  For fluorescein-conjugated antibodies, the protein concentration equals...

  IgG (mg/ml) = [A(280) - 0.31 * A(495)]/1.4]

  Then calculate the F/P ratio...

  For IgG: 3.1 * A(495)/[A(280) - 0.31 * A (495)]

  Good conjugations can range from an F/P ratio of 3 to 10.

Information for calculating F:P ratios is from Mario Roederer at NIH.